

Acacia honey from different altitudes: total phenols and flavonoids, laser-induced fluorescence (LIF) spectra, and anticancer activity

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Abstract

Objective: To investigate Acacia honey from different altitudes regarding total phenols and flavonoids, laser-induced fluorescence (LIF) spectra and anticancer activity against human cancer cell lines.

Methods: Anticancer activity was investigated using sulforhodamine B cytotoxicity assays in the following human cancer cell lines: HCT116 (colon); MCF7 (breast), and HepG2 (liver). Total phenols and flavonoids were measured using spectrophotometric methods and LIF was used to differentiate between low and high-altitude honey.

Results: The LIF spectra differed between low and high-altitude Acacia honey. High altitude Acacia honey was characterized by significantly lower total phenol content (81.47 ± 1.25 mg gallic

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acid equivalent [GAE]/100 g) and increased total flavonoids (10.63 ± 0.53 mg quercetin equivalent [QE]/100 g) versus low altitude Acacia honey (91.33 ± 0.96 mg GAE/100 g and 8.78 ± 0.23 mg QE/100 g, respectively). Low altitude Acacia honey displayed increased IC_{50} values against HCT116 and MCF7 cells (264.17 ± 10.5 and 482.65 ± 20.3 μ g/ml, respectively) versus high altitude Acacia honey (117.99 ± 12.7 and 189.82 ± 15.8 μ g/ml, respectively).

Conclusions: High altitude Acacia honey had significantly more effective anticancer activity against HCT116 and MCF7 cells compared with low altitude honey.

Keywords

Bee honey, high and low altitudes, HCT116, MCF7, IC_{50} , arbitrary units

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Introduction

Honey is a natural substance produced by honeybees from plant nectar and secretions, and from the excretions of plant sucking insects (raw materials). Honeybees collect these raw materials, add some chemicals of their own, such as carbohydrates, enzymes, and vitamins, and leave the mixture in honeycombs to mature.¹ The colour of honey ranges from clear to dark amber and it is found in four states; liquid, viscous, crystalline, and a mixture of liquid and crystalline. Chemically, honey contains many compounds, such as carbohydrates (glucose, fructose, and sucrose), enzymes (catalase and glucose oxidase), acids (gluconic and phenolic acids), proteins, flavonoids, hydrogen peroxide, dicarbonyl compounds, vitamins, and minerals.^{2,3} The composition of honey facilitates its nutritional and medicinal uses. For example, honey is used as an antimicrobial agent because of its dicarbonyl compounds (methylglyoxal) and hydrogen peroxide content, and it acts as an antioxidant because it contains flavonoids, vitamins and minerals.^{4,5}

Altitude has been reported to significantly and positively affect the concentration of many honey ingredients, such as

antioxidants, including total phenols and flavonoids, minerals, and vitamin C.⁵⁻⁷ Many studies have reported that the content of bee honey is variable regarding phenols, total flavonoids, and total phenolic acids, the content of which may be used as indicators for the botanical origin of the honey.⁸⁻¹⁷ The phenolic and flavonoids concentration in honey is associated with its antimicrobial, antitumor and antioxidant activities.^{5,12,13}

The laser-induced fluorescence (LIF) technique has proved to be useful, simple, and nondestructive for the confirmation of floral origin and authentication of bee honey.¹⁸⁻²⁰

Regarding the anticancer properties of honey, some studies have shown that honey samples have anticancer activity, while others have reported that honey is ineffective as an anticancer agent.²¹⁻²⁴

The aim of the present study was to analyse Acacia honey samples from high altitude (2246 m above sea level) and low altitude (437 m above sea level) in terms of the concentration of total polyphenols and flavonoids, the LIF spectra, and the activity against human colon cancer (HCT116), breast cancer (MCF7) and liver cancer (HepG2) cell lines.

Materials and methods

Study area

This study was conducted in the Asir region in the southwest of Saudi Arabia. The Asir region comprises an area of 80 000 km² with an estimated population of 2.19 million, and is located between the latitudes of 17.25 and 19.5 North and longitudes of 41.3 and 44.3 East.²⁵

Asir land is divided into three regions: the Tehama region, which is located between the red sea and the mountains of Alsarrah; the Alsarrah, which comprises the Alsarrah mountains that extend up to the Yemen; and a region of flat land at the eastern part. The altitude of Asir ranges from the red sea level up to more than 3 000 m above sea level at Alsouda.^{25,26} The Asir climate is moderate, with a 20–25 °C mean temperature and year-round rainfall. However, climate differences exist between the three regions of Asir according to their geographical location; near the red sea, high altitudes and the flat land in the eastern part.²⁵

The bee farms from which the honey samples were collected are located at two elevations; 437 m (05° 18' 56.51" N; 13° 42' 52.72" E) and 2246 m (05° 18' 50.52" N; 48° 42' 54.12" E; Figure 1).

Honey samples

Sample collection and treatment. Three Acacia honey samples from Alwadein (2246 m above sea level) and three Acacia honey samples from Alhabeel (437 m above sea level) were collected directly from the hives of the bee farms (to avoid adulterated honey samples) during the summer season of 2018. Three Acacia species were flowering during the collection period; *Acacia tortilis*, *Acacia ehrenbergiana*, and *Acacia origena*. The Acacia honey was separated from the honeycomb by filtration using a nylon filter.

Confirmation of floral origin. The botanical origin was confirmed microscopically as follows: 10 ml of each honey sample was diluted with 10 ml distilled water and centrifuged for 5 min at 4024.8 g. The supernatant was discarded, the pellet was mounted on a

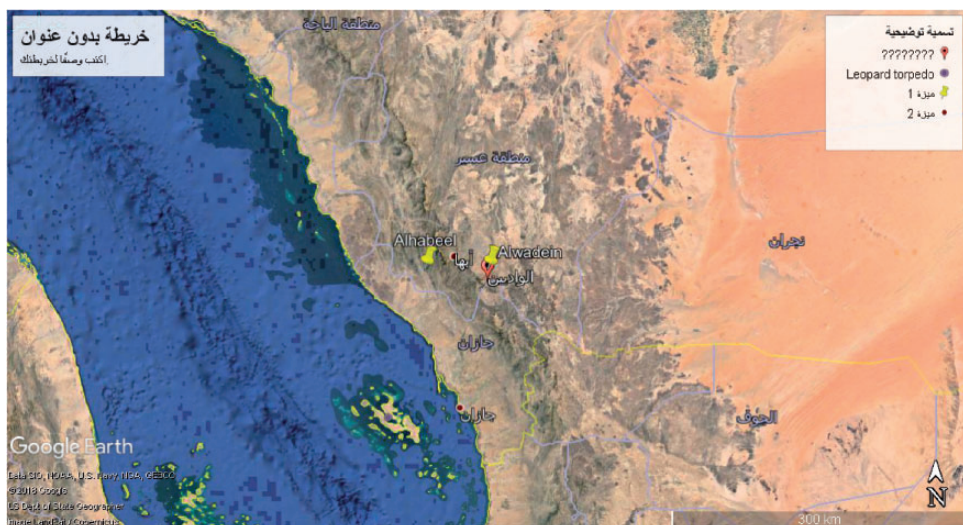


Figure 1. Satellite view of the two bee farm locations from which honey samples were collected for the present study: Alhabeel (437 m above sea level) and Alwadein (2246 m above sea level).

microscope slide, and the pollens were counted in 10 microscopic fields. Acacia pollens are characterized by circular shapes with four central monads (chambers) surrounded by eight monads. The percentage of Acacia pollens was determined according to the following equation:

$$\text{Acacia Pollen}\% = \frac{\text{number of Acacia pollens}}{\text{number of total pollens}} \times 100$$

Authentication of honey samples. Three quality parameters, namely pH, conductivity, and moisture percentage, were measured and compared to their value in the Codex Alimentarius standards, USA National Honey Board reference guide, and the gulf countries standardization organization.²⁷⁻²⁹ The pH and conductivity were determined according to the methods of the International Honey Commission.^{30,31} Moisture percentage was obtained using the oven drying method according to the Association of Official Analytical Chemists (AOAC).³²

Determination of total phenols and flavonoids

Honey sample treatment. A 5 g portion of each honey sample was placed in a 100 ml beaker, thoroughly mixed with 50 ml deionised water and magnetically stirred for 5–10 min at room temperature. The obtained honey solution was filtered through grade 1 Whatman® qualitative filter paper to obtain 0.1 g/ml honey solution, which was kept for the determination of phenolic compounds and flavonoids concentration.

Measurement of total phenols. First, 0.2N Folin–Ciocalteu reagent was prepared from 2N Folin–Ciocalteu reagent (code, 47641-100ML-F; Sigma-Aldrich; St Louis, MI, USA) as follows: 10 ml of 2N

Folin–Ciocalteu reagent were diluted with 90 ml of distilled water in a 100 ml volumetric flask. A 1 ml aliquot of each 0.1 g/ml honey sample was then added to 5 ml of 0.2N Folin–Ciocalteu reagent in a test tube. The mixture was shaken for 5 min then 4 ml of saturated sodium carbonate solution (7.5% w/v; 13418-1KG-R; Sigma-Aldrich) was added. The test tube was covered with silver foil and incubated at room temperature for 90 min with intermittent shaking. After incubation, the absorbance of the reaction mixture was measured in a spectrophotometer at 760 nm against the blank. The mean of three readings was calculated, and total phenolic content was presented as mg of gallic acid equivalent (GAE) per 100 g of honey.³³

The gallic acid standard curve was produced as follows: A stock solution of 5 mg/ml gallic acid was prepared by weighing 0.500 g of dry gallic acid (G7384-250G; Sigma-Aldrich) in a 100 ml volumetric flask and dissolving in 10 ml water: methanol solution (1: 1). Deionized distilled water: methanol solution (1: 1) was added to bring the volume up to the mark. A range of gallic acid standards (0, 0.005, 0.01, 0.02, 0.025, 0.05, 0.1, 0.2 mg/ml) were prepared using water: methanol (1: 1) solution. These standards were used to produce the calibration curve which was analysed in the same way as the honey samples.

The concentration of total phenols was calculated according to the following equation: $T = (G \times V \times 100) / M$, where T = total phenolic compounds content (mg GAE/100 g); G = concentration of gallic acid in mg/ml; V = volume of honey sample solution in ml (50 ml); and M = weight of honey sample in g (5 g).

Measurement of flavonoids concentration. First, 2 ml of a 2% aluminium chloride methanolic reagent (237051-500G; Sigma-Aldrich) was added to the same volume of honey extract solution (2 mg/ml).

After 30 min of incubation, the absorbance was read at 415 nm and compared with a blank sample consisting of a honey solution along with methanol without AlCl_3 . The total flavonoid content was calculated using a quercetin calibration curve. The mean of three readings was calculated and results were presented as mg of quercetin equivalent per 100 g of honey (mg QE/100 g).³⁴

A stock solution of 0.5 mg/ml quercetin (Q4951-100G; Sigma-Aldrich) was prepared by dissolving 0.050 g in 10 ml methanol (95%) in a 100 ml volumetric flask. Methanol was then added to bring the volume up to the final volume. A range of quercetin standards (0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.025 and 0.05 mg/ml) were prepared. Quercetin standard solutions were used to produce the calibration curve, which was analysed in the same manner as the honey samples.

The concentration of total flavonoids in honey samples was determined according to the following equation: $T = (G \times V \times 100) / M$, where T = total flavonoids content (mg QE/100 g); G = concentration of quercetin in mg/ml; V = volume of honey sample

solution in ml (50 ml); and M = weight of honey sample in g (5 g).

Laser induced fluorescence (LIF) experimental setup

The LIF experimental set up is shown in Figure 2. A diode laser (Pro100; Toptica Photonics Inc., Munich, Germany) with a maximum average power output of 29 mW at wavelength 398 nm was used as an excitation source in the experiment.

The output power of the laser beam was controlled by means of a laser control unit that changed the current and temperature of the diode. For this experiment, an average output power of 2 mW was used. The laser beam was focused to 2 mm onto one side of a quartz cuvette containing the honey sample. The emitted fluorescence radiation was collected using a lens that focused the radiation and sent it to a monochromator (ScienceTech 9055; ScienceTech Inc., London, Canada). A long pass filter (FEL0450; Thorlabs Inc., Newton, NJ, USA) was used to block the excitation laser and at the same time to pass the fluorescence radiation only. The sample holder and the monochromator were set to be

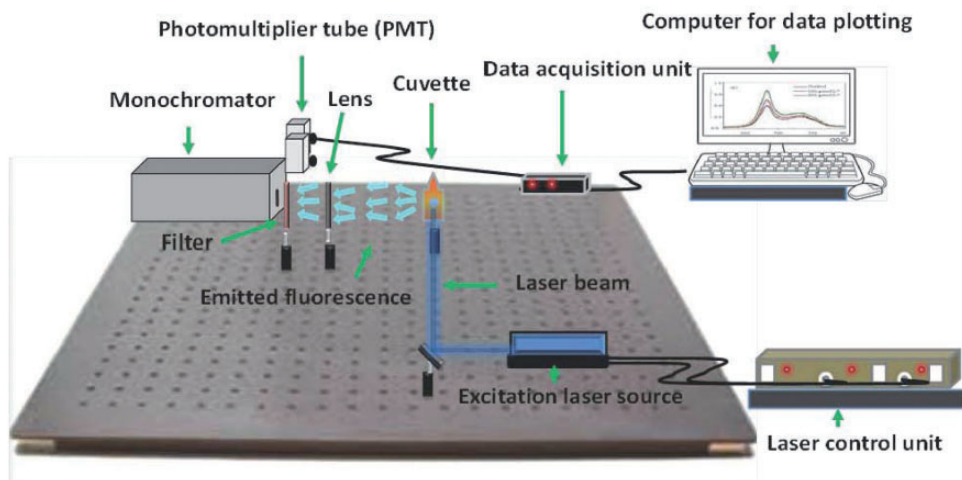


Figure 2. Schematic of laser induced fluorescence experimental setup.

perpendicular to each other. The monochromator slit was opened at a width of 0.2 mm. This arrangement offered a spectral resolution of 0.2 nm. The fluorescence signal was analysed inside the monochrome and then exited through another slit to fall on a photomultiplier tube (PMT). The PMT converted the light signal to a voltage. The signal was then passed to the data acquisition unit connected to a computer, to read and draw the signal as a function of the wavelength change (Figure 2). LIF values are presented as arbitrary units (a.u.).

Sulforhodamine B cytotoxicity assay

The anticancer activity of honey samples from two different altitudes was assessed using the calorimetric Sulforhodamine B (SRB) cytotoxicity assay, with all cells and reagents purchased from Sigma-Aldrich, as follows: Human cancer cell lines (HCT116, MCF7 and HepG2) were maintained in the appropriate optimum media (Dulbecco's Modified Eagle's Medium [DMEM]; D6429-500ML) or (Roswell Park Memorial Institute [RPMI]-1640; R0883-500ML), containing 100 U/ml penicillin (13752-5G-F), 10% v/v heat inactivated fetal bovine serum (F4135-500ML), and 100 µg/ml streptomycin (S9137-100G). Cells were grown in a humidified incubator with 5% CO₂ atmosphere at 37°C. Exponentially growing cells were collected using 0.25% w/v trypsin-EDTA (T4049-500ML) and seeded at $1-2 \times 10^3$ cells/well in 96-well plates. Cells were then treated with serial concentrations of the Acacia honey samples for 72 h and compared to untreated control cells. Doxorubicin (44583-50MG) was tested against the same cancer cell lines for comparison purposes. Cells were fixed by incubation with 10% trichloroacetic acid (TCA; T8657-250G) for 1 h at 4°C; washed three times with distilled water; subjected to 0.4% w/v SRB solution (230162-5G) for 15 min in

the dark; and then washed with 1% v/v glacial acetic acid (27225-2.5L-R). SRB-stained cells were dissolved in Tris-HCl (pH 7.4; 93313-1L) and the colour intensity was assessed at 570 nm. Dose-response curves were fitted using Sigma Plot software, version 12.0 (Systat software, San Jose, CA, USA).³⁵ The half maximum inhibitory concentrations (IC₅₀ values) were calculated for high and low altitude honey samples and for the doxorubicin positive control.

Statistical analyses

Data are presented as mean ± SD, and differences between mean values of the studied parameters in the different honey samples were statistically analysed using Student's *t*-test. All statistical analyses were performed using SPSS software, version 20.0 (SPSS Inc. Chicago, IL, USA). A *P* value ≤0.05 was considered statistically significant.

Results

All of the Acacia honey samples were found to be monofloral (more than 50% of the pollens were Acacia pollens). The pH and moisture percentage values of all the Acacia honey samples were within their ranges in the Codex Alimentarius standards and the US national honey board reference guide.^{27,28} The conductivity of the high altitude honey (1650.6 µS/cm) exceeded the Codex standards for honey (<800 µS/cm), but was within the range determined by the gulf countries standardization organization (<2000 µS/cm).²⁹ Altitude was shown to significantly affect the studied quality parameters (Table 1).

The high-altitude Acacia honey samples (from 2246 m above sea level) were characterized by a significantly lower concentration of total phenolic compounds and higher concentration of flavonoids

Table 1. Concentration of total phenol compounds and total flavonoids, and authentication parameters, in Acacia honey samples obtained from low and high altitudes.

Sample	Total phenols, mg GAE/100 g	Total flavonoids, mg QE/100 g	pH	Conductivity, $\mu\text{S}/\text{cM}$	Moisture, %
Acacia honey from 437 m	91.33 ± 0.96	8.78 ± 0.23	4.36 ± 0.08	184.6 ± 4.51	13.5 ± 0.76
Acacia honey from 2246 m	81.47 ± 1.25	10.63 ± 0.53	5.4 ± 0.05	1650.6 ± 7.15	15.7 ± 0.84
Statistical significance	$P = 0.0016$	$P = 0.031$	$P < 0.001$	$P < 0.001$	$P = 0.043$

Data presented as mean \pm SD.

GAE, gallic acid equivalent; QE, quercetin equivalent.

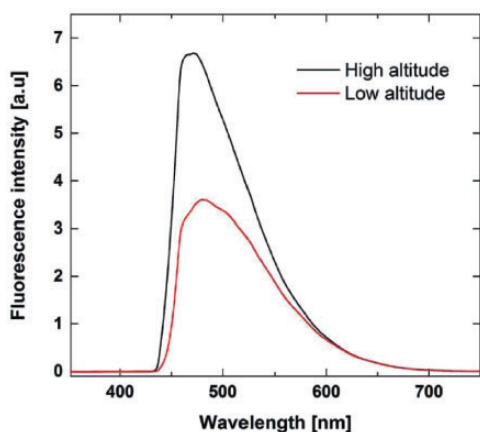


Figure 3. Laser induced fluorescence spectra of the low and high-altitude Acacia honey samples. The λ values of the high and low altitude Acacia honey were 6.68513 arbitrary units (a.u) and 3.60067 a.u, respectively.

compared with low-altitude Acacia honey samples (from 437 m above sea level; $P < 0.05$; Table 1).

The LIF λ value of high altitude Acacia honey was 6.68513 a.u at 471.3 nm, while the λ value of low altitude Acacia honey was 3.60067 a.u at 479.45 and 479.5 nm (Figure 3). In terms of total phenols and flavonoids, there was a similar trend between flavonoids and fluorescence intensity, in that they were both higher in the high-altitude honey samples, suggesting that the fluorescence may be due to the flavonoid concentration (Table 1) and (Figure 3). This suggests that LIF may be effective in differentiating between low and

high-altitude honey samples from the same floral origin.

The SRB cytotoxicity assay revealed that Acacia honey samples were toxic against all of the cancer cell lines, shown by the IC_{50} values (Table 2). The Acacia honey samples from the 2246 m altitude had significantly higher anti-HCT116 and anti-MCF7 activity compared with the honey samples from the 437 m altitude, shown by statistically significant differences in IC_{50} values ($P < 0.001$; Table 2). There was no statistically significant difference in cytotoxicity against HepG2 cells between honey samples from the two different altitudes (Table 2). Doxorubicin displayed the lowest IC_{50} values against all of the cancer cell lines.

Discussion

In the current study, the mean values for total phenolic compounds in Acacia honey samples from high altitude (2246 m) were significantly lower than values from low altitude (437 m), being 81.47 ± 1.25 and 91.33 ± 0.96 mg GAE/100 g, respectively. Conversely, the high-altitude Acacia honey contained an increased concentration of total flavonoids (10.63 ± 0.53 mg QE/100 g) compared with low altitude Acacia honey (8.78 ± 0.23 mg QE/100 g). The significant differences between the low and high-altitude Acacia honey samples may be due to climate differences between low and high altitudes. In a previously published paper, the present authors showed

Table 2. Cytotoxicity (IC₅₀) of honey samples from high and low altitudes, and doxorubicin, against three human cancer cell lines.

Human cell line	Honey sample IC ₅₀ value, µg/ml		Statistical significance	Doxorubicin IC ₅₀ value, µg/ml
	High altitude	Low altitude		
HCT116	117.99 ± 12.7	264.17 ± 10.5	P = 0.0006	1.2 ± 0.036
MCF7	189.82 ± 15.8	482.65 ± 20.3	P = 0.0002	1.09 ± 0.044
HepG2	150.93 ± 19.1	173.05 ± 25.6	NS	0.92 ± 0.046

Data presented as mean ± SD.

NS, no statistically significant between-group difference ($P > 0.05$, Student's *t*-test).

that high altitude is characterized by disintegrated pollens, which may contribute to the significantly different physicochemical properties and chemical composition of honey samples.³⁶ Furthermore, differences between the low and high altitude honey may be due to the presence of pollens other than Acacia pollens, and to the fact that bees living at high altitude depend partly on plant and insect secretions, which leads to the production of honeydew beside the blossom honey.³⁷

Previous studies have reported variable results regarding the concentration of total phenols and flavonoids in honey samples. For example, three studies reported that the total flavonoids concentration in Yemeni honey of different botanical origins was 122–5482 µg/100 g and 261–1646 µg/100 g,^{13–15} and the concentration of total flavonoids in the Yemni honey was less than that of the honey samples of the present study, which may be due to the different floral origins. The Yemni honey research studied polyfloral and cactus honey samples, while the present study investigated total flavonoids in Acacia honey samples.^{13–15} Pontis et al.³⁸ reported total flavonoids in honey samples from Brazil that ranged from 9 to 4.8 mg QE/kg, which was very low compared with the findings of the present study. Omani honey samples registered high concentrations of total phenols and total flavonoids compared with the findings of the present

study, ranging from 162.4–289.8 mg GAE/100 g and 161.3–289.0 mg QE/100 g, respectively.³⁹ Similar to the present findings, Cabrera et al.⁴⁰ measured total phenols and flavonoids in Acacia honey samples from Burkina Faso, and reported concentrations of 93.4 ± 0.87 mg GAE/100 g and 6.14 ± 0.35 mg QE/100 g, respectively. Another Saudi study reported comparable total phenols in Acacia honey samples, ranging from 74–84 mg GAE/100 g.⁴¹ A comparison between the present study findings and those of previously published studies regarding the concentration of total phenols and flavonoids in honey samples is presented in Table 3.

A search of the literature did not reveal any published articles regarding the use of LIF as a technique to differentiate between low and high-altitude honey samples. However, two previous studies showed the possibility of using spectra obtained with the LIF technique for detecting honey adulteration and in honey authentication.^{18,20} In addition, Ruoff et al.¹⁹ showed that the laser fluorescence spectra of bee honey may be used for determining bee honey's floral origin. The results of the present study suggest that LIF may be used for differentiating between low and high-altitude honey samples because of their flavonoid concentration, and not the total phenols.

It is well known that dark honeys are characterized by their high content of polyphenols and flavonoids, which may be the

Table 3. Comparison between the concentration of total phenols and flavonoids in honey samples from the present research and from previously published studies.

Study	Total phenols	Total flavonoids	Botanical origin	Geographical origin
Present study				
Low altitude honey (437 m)	91.33 ± 0.96 mg GAE/100 g	8.78 ± 0.23 mg QE/100 g	Acacia	Saudi Arabia
High altitude honey (2 246 m)	81.47 ± 1.25 mg GAE/100 g	10.63 ± 0.53 mg QE/100 g	Acacia	Saudi Arabia
Akbari et al. (2020) ¹⁶	33.21 ± 5.64 mg/100 g	2.71 ± 0.17 mg/100 g	Acacia	Iran
Al-Farsi et al. (2018) ³⁹	1624–2898 mg GAE/kg	1613–2890 mg QE/kg	Acacia (Sumer), Ziziphus and multiflora	Sultanate of Oman
Cabrera et al. (2017) ⁴⁰	93.4 ± 0.87 mg GAE/100 g	6.14 ± 0.35 mg QE/100 g	Acacia	Burkina Faso
Alqarni et al. (2016) ⁴¹	0.74–0.84 mg GAE/g	–	Acacia	Saudi Arabia
Ahmed et al. (2016) ¹⁴	–	122–5482 µg/100 g	Polyfloral and Cactus	Yemen
Ahmed et al. (2014) ¹³	–	261–1646 µg/100 g	Ziziphus jujuba	Yemen
Pontis et al. (2014) ³⁸	–	9–4.8 mg QE/kg	Multifloral	Brazil

Data presented as mean ± SD, or range.

GAE, gallic acid equivalent; QE, quercetin equivalent.

Differences in total phenols and flavonoids concentrations may be due to different botanical or geographical origin.

cause of their antioxidant and anticancer capacities.^{38–40} Previous research has shown that bee honey has variable *in vitro* effects on cancer cell lines depending on the plant source. For example, some types of honey have anti-proliferative effects against human cancer cell lines, such as thyme honey, while others (e.g. Fir honey) have been shown to stimulate the viability of human cancer cell lines, such as MCF7.^{21–24} In the present study, Acacia honey from both high and low altitudes were shown to exhibit cytotoxic effects against three human cancer cell lines. High altitude honey displayed increased cytotoxicity against HCT116 and MCF7 cells, and also contained increased levels of flavonoids, versus the low altitude honey.

The results of the present study are limited due to the small number of honey samples obtained from only two locations. Thus, it is not possible to generalize the conclusions, and further research is required to validate the results.

The present study has provided a basis for further research on the effect of altitude on the physicochemical properties and biological activities of honey samples. However, the major findings of this study are that: (1) High-altitude Acacia honey may have higher activity against human cancer cell lines compared with low altitude Acacia honey; and (2) The laser induced fluorescence technique may be used as a simple, rapid, and nondestructive technique for differentiating between low and high-altitude honey samples, because of the flavonoid content of the honey rather than the total phenols content.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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